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Inhibition of Dopamine and Choline Acetyltransferase Concentrations in Rat CNS Neurons by Rat α_1- and α_2-Macroglobulins

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Previous studies have implicated human alpha-2-macroglobulin (α_2M) as a potential regulator of neuronal development and function. Rat alpha-1-macroglobulin (α_1M) and acute-phase alpha-2-macroglobulin (α_2M) are murine homologues of human α_2M. In this report, we tested the effect of intracranially infused serotonin-activated rat α_1M (5HT-α_1M) on the concentration of dopamine (DA) in the corpus striatum in vivo and the effect of 5HT-activated rat α_1M and α_2M on the choline acetyltransferase (ChAT) activity upon embryonic basal forebrain neurons in culture. The results show that direct infusion of 0.65 nmole rat 5HT-α_1M into the adult rat corpus striatum produced a consistent attenuation upon striatal DA concentrations. This decrease was particularly prominent at 5–7 days post-infusion. In addition, rat 5HT-α_1M and rat 5HT-α_2M, like human 5HT-α_2M, all significantly inhibited ChAT activity of embryonic rat cerebral cortex neurons. Although normal human α_2M and rat α_2M were either marginally or insignificantly inhibitory in this preparation, normal rat α_1M dose-dependently inhibited ChAT activity. These results demonstrate that monoamine-activated α-macroglobulins from rat depress dopaminergic and cholinergic neurotransmitter systems in the CNS, and this suggests a potential regulatory role of these alpha-macroglobulins in neurotransmitter metabolism. J. Neurosci. Res. 51:541–550, 1998. © 1998 Wiley-Liss, Inc.

Key words: acetylcholine; Alzheimer's disease; monoamines; nigrostriatum; neurotransmitter; neurotrophin; Parkinson's disease

INTRODUCTION

Rat alpha-1-macroglobulin (α_1M) and alpha-2-macroglobulin (α_2M) are high-molecular-weight glycoproteins (mol. wt. = $7.05–7.3 \times 10^5$), which are structurally and functionally homologous to human α_2M and pregnancy zone protein (PZP). This α_2M family of proteins are proteinase inhibitors and possess internal thioester bonds (Swenson and Howard, 1979; Sand et al., 1985; Lonberg-Holm et al., 1987a). The physiological role of thioester bonds in α_2M homologues is unclear, but they are known to either react with entrapped proteinases or be auto-hydrolyzed subsequent to peptide bond cleavage on the α_2M site by the entrapped proteinase (Sottrup-Jensen et al., 1981; Eggertsen at al., 1991). In addition, nucleophilic amines such as methylamine and serotonin readily react with thioester bonds to form covalently linked conjugates (Swenson and Howard, 1979; Liebl and Koo, 1993a). Such proteinase and amino complexes exhibit faster electrophoretic mobilities (Barrett et al., 1979) and are capable of binding to α_2M-receptors (α_2M-R/LRP), which occur in various cell types including macrophages, neurons, and astrocytes (Moestrup et al., 1992). Hence amine-modified or proteinase-reacted α_2M is also called the "activated" or "fast" form of α_2M to differentiate it from the normal α_2M (N-α_2M), which lacks such properties.

Rat α_1M occurs naturally at high concentrations in the plasma ($>$ 1 mg/ml) (Gordon, 1976) and its mRNA is expressed abundantly in such diverse tissues as the brain, kidney, lung, liver, spleen, pancreas, and thymus (Eggertsen et al., 1991). Rat α_2M, on the other hand, occurs only in trace amounts in tissues under normal conditions, but its concentration is greatly elevated during inflammation ($>$ 2 mg/ml in plasma); hence it is an acute-phase protein (Ganrot, 1973; Schaeufele and Koo, 1982; Lonberg-Holm et al., 1987b). α_1M shares 57% overall amino acid sequence identity with rat α_2M (Eggertsen et al., 1991), but rat α_2M is much more similar to human α_2M in both

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antigenic property and subunit structure than α_1M, albeit human α_2M is not an acute-phase protein (Schaeufele and Koo, 1982). Aside from some structural and functional characteristics, essentially nothing is known regarding the physiological roles of these rat macroglobulins.

Recently both normal N-α_2M and monoamine-activated α_2M (MN-α_2M) of the mouse and the human have been shown to carry neurotrophins and other cytokines, presumably to protect the carried molecules from tissue proteinases (Koo and Stach, 1989; Koo and Liebl, 1992; Liebl and Koo, 1993b; Wolf and Gonias, 1994). In addition, human MN-α_2M can dose-dependently inhibit a) neurite outgrowth and survival of embryonic central nervous system (CNS) and peripheral nervous system (PNS) neurons (Koo and Liebl, 1992; Liebl and Koo, 1993a), b) choline acetyltransferase activity of basal forebrain neurons (Liebl and Koo, 1994), c) dopamine (DA) release by adult caudate putamen (Hu et al., 1994, 1996a), d) the development and maintenance of long-term potentiation in adult rat hippocampal slice (Çavuş et al., 1996), and e) *trk* receptor activation and signal transduction presumably via its binding to *trk* (Koo and Qiu, 1994). Human N-α_2M, on the other hand, has little or no such effects. Rats have two α-macroglobulin homologues, and rat MN-α_2M also inhibits neurite outgrowth and ChAT activity of embryonic cortical neurons (Koo et al., 1994). But it is not known if α_1M also has similar neuro-inhibitory properties as human and rat α_2M.

In this report, we employed the homologous rat system to test the effect of rat macroglobulins on the DA concentrations in adult rat corpus striatum (CS) in vivo and the choline acetyltransferase (ChAT) activity of embryonic rat basal forebrain cells in vitro. These two test systems were chosen for the following reasons: a) Both DA and ChAT levels in the brain have been shown to be adversely affected by MN-α_2M (Hu et al., 1994; Liebl and Koo, 1994; Koo et al., 1994; Hu et al., 1996a); b) the losses of DA in CS and acetylcholine in the striatum and basal forebrain are major abnormalities associated with Parkinson's and Alzheimer's diseases, respectively; and c) the inflammatory process and its by-products in the brain (including α_2M in the senile plaques) have been implicated as potential causes of neurodegenerative diseases such as Alzheimer's (Strauss et al., 1992; Liebl and Koo, 1994; Stewart et al., 1997). Our results demonstrate that the activated form of α_1M, like human MN-α_2M, also suppresses the two neurotransmitter systems. One surprising finding is that even rat N-α_1M can also dose-dependently inhibit ChAT activity of cholinergic neurons; this suggests a potential regulatory role for α_1M in the CNS.

MATERIALS AND METHODS

Animals

Male (N=30, 275–300 gm body weight) adult Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were used in the present experiment. All animals were housed individually in plastic cages under a 12:12 hr light:dark cycle with lights on at 06:00 hr. Food and water were available ad libitum. All treatments used in this experiment adhere to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee at the College of Medicine.

Rat Alpha-Macroglobulin Purification and Activation by Monoamines

Rat α_1M and α_2M were, respectively, prepared from normal and acute-phase plasma as previously described with slight modifications (Schaeufele and Koo, 1982). Briefly, to obtain acute-phase plasma, adult rats (Sprague-Dawley, 250–400 gm) were injected intramuscularly with turpentine and bled 2 days later. Normal and turpentine-treated rats were bled by cardiac puncture and the blood was collected into a syringe containing about 1/9 volume 3.8% sodium citrate in 2 mg/ml soybean trypsin inhibitor. The blood and the plasma were kept in plastic containers on ice throughout the preparation. Rat plasma was obtained from the blood by centrifugation (1,200g, 30 min). The plasma was treated with barium chloride and barium sulfate, and then fractionated by 4–12% polyethylene glycol in ice. Subsequently, the precipitate was dissolved in 0.01 M sodium borate, pH 8.0, buffer. The sample was applied onto a Blue-Sepharose column equilibrated with the same buffer. The α-macroglobulin peak was pooled and then applied onto a Whatman DE-52 column also equilibrated with the same borate buffer. The column was eluted successively with the following: 0.01 M sodium borate, pH 8.0, buffers containing, respectively, 0.05, 0.08, 0.10, and 0.15 M NaCl. Rat α_1M and α_2M were, respectively, eluted by the 0.08 and 0.15 M NaCl buffers from the DE-52 column. The α-macroglobulins were pooled, dialyzed into phosphate-buffered saline (PBS), and sterilized by filtration (Acrodisc 0.22 μm syringe filter, Gelman Sciences, Inc., Ann Arbor, MI) before used in bioassays (Hudson and Koo, 1982). The presence of α_1M and α_2M in each column fraction was detected by double immunodiffusion using rabbit anti-rat α_1M and anti-rat α_2M antibodies, respectively (Schaeufele and Koo, 1982).

Methylamine- or serotonin-activated α_1M and α_2M (MA-α_1M, 5HT-α_1M; MA-α_2M, 5HT-α_2M) were obtained by incubating the purified normal rat α-macroglobulin with 0.04 M methylamine or serotonin (in 0.2 M

Tris-HCl buffer, pH=8.1) for 2.5 hr at 37°C, and then dialysized with PBS. The purity and concentration of an α-macroglobulin preparation were determined as described previously (Schaeufele and Koo, 1982). The extent of modification of each protein was determined by trypsin binding assay (Koo and Liebl, 1992).

Surgical Methods and General Procedures

The animals received a unilateral infusion of 5HT-α_1M into one side of the corpus striatum (CS) (right or left) and a unilateral infusion of its PBS vehicle into the contralateral side. The sides of the CS receiving 5HT-α_1M or PBS were alternated for each animal. Infusions were performed while the animals were under a ketamine: acepromazine anesthesia (10:1) at a dose of 100 mg/kg i.p., with supplemental doses administered as required. The infusion cannula (30 g) was inserted by using a stereotaxic apparatus with coordinates derived from the DeGroot rat brain atlas (1959). Cannula placement was directed toward the rostral dorsal area of the CS using the coordinates 2.3 mm anterior to bregma, 2.5 mm lateral from the superior sagittal sinus, and to a depth of 4.4 mm from dura. The 5HT-α_1M was maintained in PBS (pH 7.2) at a concentration of 35.4 mg/ml. Infusions (5HT-α_1M or PBS) were performed over a 20 min period by using a peristaltic pump at a flow rate of 0.667 μl/min. Rats were divided into five groups and sacrificed respectively on days 1, 2, 3, 5, and 7 following infusions. The animals were sacrificed by rapid decapitation and the CS from each side of the brain was dissected. Following a midline incision, the bisected brain was separated. The ventricles on the medial side of the brain were pried open and the cortex cut away revealing the CS. The perimeter of the CS within the corpus callosum, anterior commissure, and internal capsule/cortex was cut and the CS removed using curved scissors. The tissue was weighed and placed in 500 μl of cold 0.1 N perchloric acid. Subsequently, tissue samples were sonicated, centrifuged, and an aliquot removed for determination of dopamine (DA). In addition to the CS, the olfactory tubercle (OT) was also dissected bilaterally from each animal and prepared for determination of DA concentrations. Dissection and assay of OT DA concentrations served as a control to evaluate the amount of variation between bilateral estimations of DA concentrations attributable to factors involved in dissection and assay of tissue concentrations. The evaluation for the effects of foreign protein infusions into the CS has been performed previously by using bovine serum albumin (BSA) vs. PBS infusions (Hu et al., 1994).

Dopamine Assay

Tissue samples were assayed for DA using a high-performance liquid chromatography system with electrochemical detection (ESA Inc., West Lafayette, IN). Biogenic amines were separated on a biophase C-18, 5 μm sphere column (Bioanalytical Systems, Inc.). The mobile phase consisted of 50 mM sodium acetate, 27.4 mM citric acid, 10 mM EDTA, and 7% methanol in filtered distilled water. The final pH of 4.5 was achieved with the addition of sodium hydroxide and the mobile phase was filtered (0.45 μm filter, Millipore Corp., Bedford, MA) prior to use. Standards for DA were stored in 0.1 N perchloric acid and doses of 31, 62, 125, 250, and 500 pg/20 μl were used to construct a standard curve. The sensitivity for DA as defined by a response three times above baseline noise was between 15.5 and 31 pg.

Choline Acetyltransferase (ChAT) Assay

ChAT assays were performed on embryonic rat basal forebrain cells, which were obtained from timed pregnant rat (E-15) under sterile conditions. Frontal cortices were dissected from eight to ten embryos, the tissues were dissociated by trypsinization, and the cells were combined. An aliquot containing 1×10^7 cells was added to each 35 mm Petri plate (Falcon, Oxnard, CA) containing fresh growth media [Eagle's minimal essential medium, containing 10% fetal bovine serum, 0.1% D-glucose, 2 mM L-glutamine, and 50 μg/ml gentamicin (Sigma Chemical Co., St. Louis, MO)] (Liebl and Koo, 1993a). The cells (1×10^7) were then separately incubated with PBS, BSA, rat α_1M, α_2M, 5HT-α_1M, or 5HT-α_2M in a final volume of 2 ml for 3 days at 37°C in a humidified 5% CO_2 atmosphere. The effect of rat α_1M, α_2M, 5HT-α_1M, and 5HT-α_2M on ChAT activity was then determined as described by Fonnum (1975) and compared to that of BSA controls carried out in parallel under the same culture conditions as described by Liebl and Koo (1994). Briefly, following the completion of a desired incubation time, the forebrain cortical cells in each culture plate were homogenized in 100 μl PBS. The homogenates, 25 μl in triplicate, were treated with 4 μl of 10% Triton X-100 to release ChAT. An additional 10 μl aliquot was stored at −20°C for protein determination (Lowry et al., 1951). A 50-μl aliquot of a solution containing 0.2 nmole [^{3}H]acetyl coenzyme A (specific radioactivity = 437,440 dpm/nmole; New England Nuclear, Boston, MA), 8 mM choline bromide, 300 mM sodium chloride, 20 mM EDTA, and 0.1 mM physostigmine in 50 mM sodium phosphate buffer at pH 7.4 was then added to the homogenate-triton mixture. After 30 min incubation at 37°C, the mixture was washed with cold PBS and transferred to a scintillation vial. To each

vial was added 2 ml of 15 mM tetraphenylboron in acetonitrile and 8 ml of a toluene scintillation mixture (6% PPO and 0.02% POPOP). The vial was briefly shaken to allow the radioactive acetylcholine to be separated into the toluene phase. Each sample was counted for radioactivity and the ChAT activity expressed as pmoles/min/mg protein.

Statistical Analysis for Dopamine Assay

Concentrations of DA, expressed as pg of DA per mg of tissue weight, were compared between CS sides infused with 5HT-α_1M vs. PBS for each group of animals sacrificed on days 1, 2, 3, 5, and 7 post-infusion. A 2 $\times$ 5 (Treatment $\times$ Day of Sacrifice) independent groups two-way ANOVA was used to analyze these data. The Fisher's Least Significance Test was used to analyze Post-hoc difference between 5HT-α_1M vs. PBS sides on each day with a $P < 0.05$ required for results to be considered statistically significant. An identical analysis was applied to the OT data. The significance of the ChAT data were calculated according to the Student's t-test.

RESULTS

The Effect of 5HT-α_1M on Dopamine Concentrations in CS in Vivo

The effect of 5HT-α_1M vs. PBS infusion in vivo upon DA concentrations (pg/mg) from CS along with the corresponding determinations from OT are presented in Figure 1A,B. The overall values (mean $\pm$ SEM) of the total DA concentrations from CS or OT of the animals dissected at days 1, 2, 3, 5, and 7 following infusions are shown in line graphs. Analysis of the CS data shows an overall significant reduction in DA concentration on sides of the CS infused with 5HT-α_1M ($F_{[1,50]} = 24.85$, $P < 0.0001$). An evaluation by post-hoc Fisher's Least Significance Test revealed that CS DA concentrations on sides infused with the 5HT-α_1M were significantly lower than contralateral PBS infused sides on days 2, 5, and 7 post-infusion. No significant differences between the two CS sides were obtained at days 1 or 3 post-infusion.

No statistically significant treatment effects ($F_{[1,50]} = 0.90$, $P = 0.35$) or interactions ($F_{[4,50]} = 0.42$, $P = 0.79$) were obtained for the OT DA concentrations. There was an overall statistically significant effect for the day of sacrifice with OT DA concentration levels on day 7 being greater than that observed on days 1,3, and 5.

The DA concentrations from sides of the CS infused with 5HT-α_1M versus PBS-treated contralateral controls were also compared, each with respect to the mean DA concentration of both sides of OT for each animal (Fig. 2). Analysis of these data (in ratios of (DA of CS)/(DA of OT) again shows an overall significant reduction in DA from the 5HT-α_1M-treated sides in comparison with the PBS-treated sides of the animals dissected at the same five time periods ($P < 0.05$).

The Effect of α_1M, α_2M, 5HT-α_1M, and 5HT-α_2M on ChAT Activity

The effect of rat alpha-macroglobulins on ChAT expression was determined by cholinergic basal forebrain neuron culture. Rat normal α_2M and 5HT-α_2M were compared to human α_2M and BSA in 3-day-old cultures containing 1×10^7 cells and 0.75 mg/ml of test proteins. The results obtained from the PBS vs. BSA cultures were not significantly different from each other, so all the α-macroglobulin results were compared to and calculated with respect to the BSA controls. Figure 3 shows that human 5HT-α_2M (H:5HT-α_2M) produced a 40% decrease in ChAT activity, while normal human α_2M (H:α_2M) produced only a 16% reduction. Rat 5HT-α_2M (R:5HT-α_2M) produced a similar effect as human 5HT-α_2M at the same α_2M concentration, having a 36% decrease in ChAT activity. Normal rat α_2M (R:α_2M) produced a 10% decrease in ChAT activity. Furthermore, rat 5HT-α_2M at the 0.25 mg/ml concentration only produced a 17% decrease in ChAT expression, which correlates with the 20% decrease produced by 0.25 mg/ml human 5HT-α_2M (Liebl and Koo, 1994). The above results suggest that both rat and human 5HT-α_2M are similar in their abilities to inhibit ChAT activity.

The effect of rat normal α_1M and 5HT-α_1M on ChAT expression has also been studied. Figure 4 shows that normal rat α_1M could dose-dependently decrease ChAT activity after 3 days of incubation, with rat α_1M at 0.25, 0.75, and 1.5 mg/ml, all producing statistically significant dose-dependent decreases (24%, 47%, and 66% decreases, respectively) in ChAT activity. 5HT-α_1M also produced a 62% decrease in ChAT activity. Although both normal and 5HT-α_1M are inhibitory, 5HT-α_1M is slightly more active (i.e., 47 vs. 62%). Such ChAT

Fig. 1. **A:** Comparisons of dopamine (DA) concentrations (pg-DA/mg-tissue weight) from sides of the corpus striatum (CS) infused with 472.2 µg (0.65 nmole) of rat 5HT-α_1M in the PBS vehicle vs. PBS only treated contralateral CS (control). Values represent the mean $\pm$ SEM from rats sacrificed at 1, 2, 3, 5, and 7 days post-infusion with N=6 rats at each day. Concentrations of DA from CS sides infused with rat 5HT-α_1M were significantly lower ($P < 0.05$) than untreated contralateral sides at 2, 5, and 7 days post-infusion. **B:** Comparisons of DA concentrations from the corresponding olfactory tubercle (OT) of the animals described in A. No statistically significant differences between the two sides of OT were obtained on any of the days sampled.

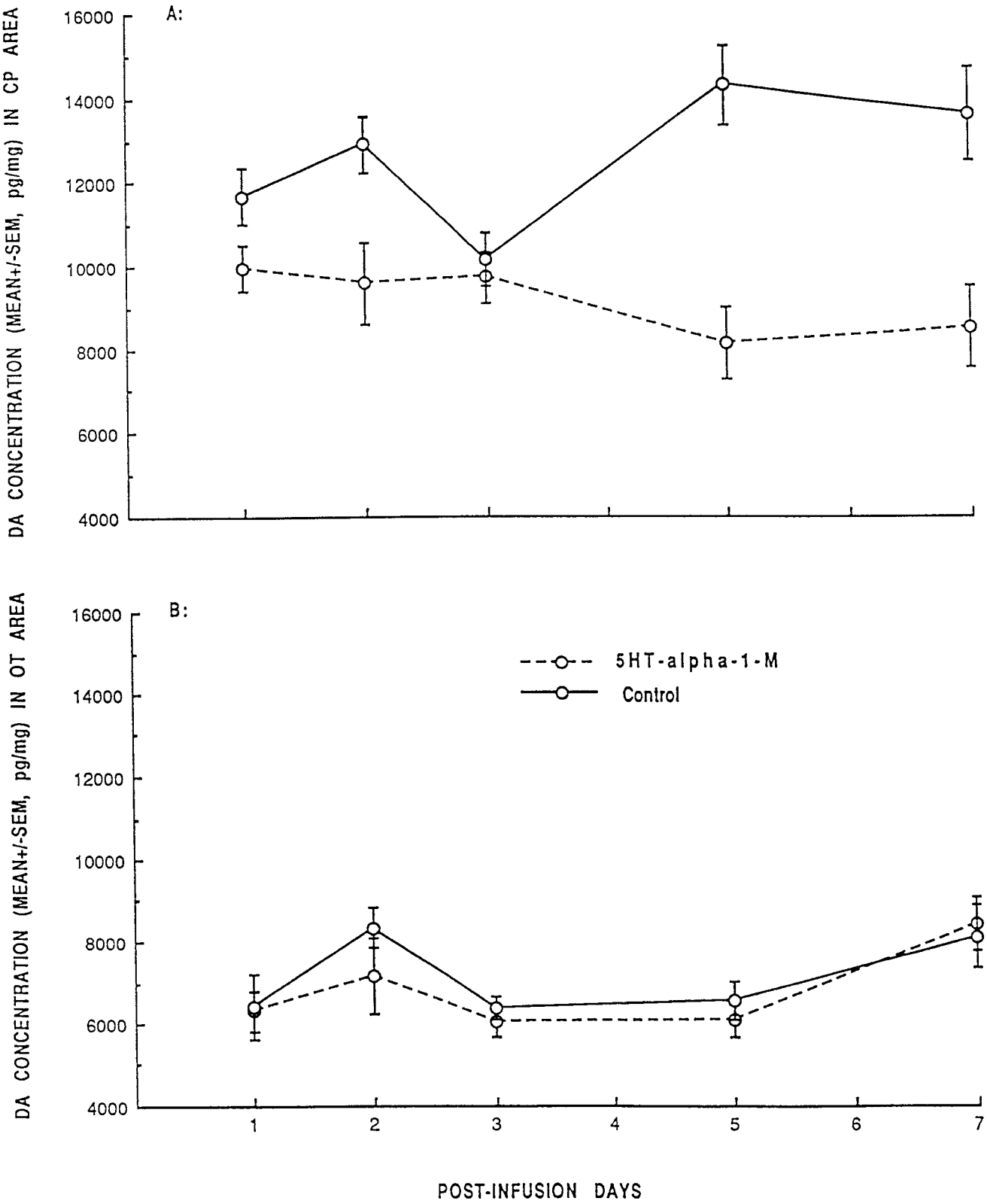
A:
DA CONCENTRATION (MEAN+/-SEM, pg/mg) IN CP AREA
16000
14000
12000
10000
8000
6000
4000
B:
DA CONCENTRATION (MEAN+/-SEM, pg/mg) IN OT AREA
5HT-alpha-1-M
Control
1
2
3
4
5
6
7
POST-INFUSION DAYS

Figure 1.

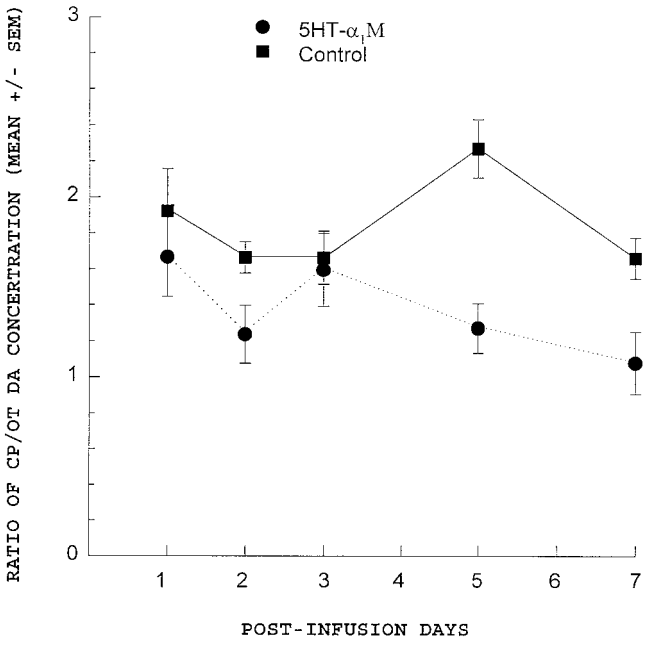

Fig. 2. Comparisons of the dopamine (DA) concentrations from sides of the corpus striatum (CS) infused with 472.2 µg (0.65 nmole) of rat 5HT-α_1M in the PBS vehicle vs. PBS only treated contralateral CS (control) as expressed by the ratio of CS DA/OT DA concentrations of the corresponding animals. The overall mean OT DA concentration [(left + right)/2] for each animal was used in these calculations since no differences in OT DA concentrations were obtained between 5HT-α_1M-treated and control sides. Values represent the mean ± SEM of the ratio of CS DA concentrations/OT mean DA concentration from rats sacrificed at 1, 2, 3, 5, and 7 days post-infusion with N=6 rats at each day. Overall concentrations of DA from CS sides infused with rat 5HT-α_1M were significantly lower ($P <$ 0.05) than untreated contralateral sides. In addition the ratios of DA from CS sides infused with rat 5HT-α_1M were significantly lower than contralateral sides infused with PBS at 2, 5, and 7 days post-infusion.

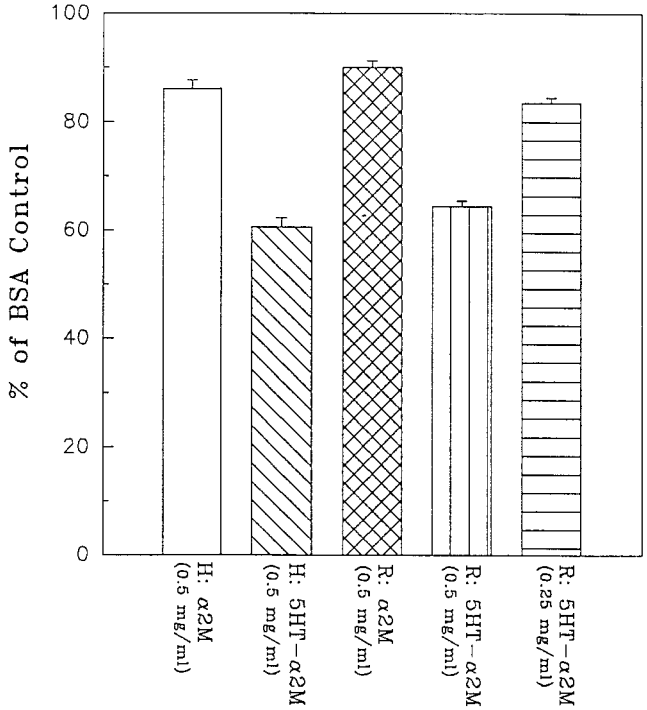

Fig. 3. The percent change in the ChAT activity of cholinergic basal forebrain cultures containing human (H) and rat (R) normal α_2M and 5HT-α_2M, as compared to the ChAT activity in the BSA control. BSA (0.75 mg/ml) or 5HT-α_2M (0.25 and 0.5 mg/ml) was incubated with 1×10^7 cells for 3 days at 37°C in a 5% CO_2 humidified atmosphere as described in the methods. The ChAT activity of the BSA control was 190 pmoles/min/mg protein, which was used as a reference standard representing 100% ChAT activity. With respect to the BSA control (as 100%), statistically significant differences were obtained for the human normal α_2M (0.5 mg/ml, $P<0.05$), human 5HT-α_2M (0.5 mg/ml, P_2M (0.5 mg/ml, $P<0.005$), rat normal α_2M (0.5 mg/ml, $P<0.2$), and rat 5HT-α_2M (0.5 mg/ml, $P<0.005$).

inhibition was not due to cell loss, since there was no visible decrease in cell viability in all the cultures seeded at high cell numbers (such as 1×10^7 cells/plate) even after 6 days of culture. We conclude from the above data that both rat MN-α_1M and MN-α_2M, like human MN-α_2M, are inhibitory to dopaminergic and cholinergic neurotransmitter systems, but it is particularly surprising to find rat normal α_1M also being dose-dependently inhibitory to ChAT, since all our data to date show normal α_2M from rat and human are only marginally inhibitory or not at all (Liebl and Koo, 1994; Koo et al., 1994; Hu et al., 1996a).

DISCUSSION

Human MN-α_2M has been previously shown to consistently and dose-dependently inhibit the functions of all the CNS and PNS neurons and in vitro cell lines tested, whereas N-α_2M is generally inactive (Koo et al., 1994; Çavuş et al., 1996; Hu et al., 1996a,b). Our preliminary report also demonstrated that MN-α_2M of the rat is also inhibitory to the central neurons (Koo et al., 1994). Hence the effect of α_2M on the nervous system has been quite established in various in vitro and in vivo systems, but the effect of α_1M on neurons has never been studied. Therefore, in this report we primarily investigated the effect of α_1M on two CNS neurotransmitter systems. In addition, we expanded our investigation in rat α_2M and postulate its potential role in CNS neurodegenerative diseases.

Our previous studies have shown that human methylamine-activated α_2M induces an acute-phase initial burst increase of DA release in vitro and a final long-term decrease in CS DA concentrations (Hu et al., 1994,

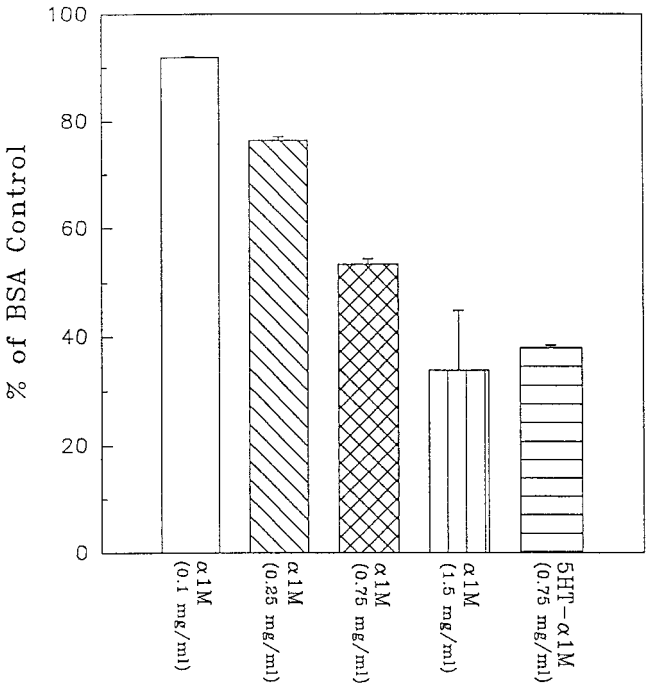

Fig. 4. The percent change in the ChAT activity of cholinergic basal forebrain cultures containing rat normal α_1M or 5HT-α_1M as compared to the ChAT activity in the BSA control. BSA (0.75 mg/ml), normal α_1M (0.1, 0.25, 0.75, and 1.5 mg/ml), or 5HT-α_1M (0.75 mg/ml) was incubated with 1×10^7 cells for 3 days at 37°C in a 5% CO_2 humidified atmosphere as described in the methods. The ChAT activity of the BSA control was 107.4 pmoles/min/mg protein, which represents 100% ChAT activity. With respect to the BSA control (as 100%) statistically significant differences were obtained for the α_1M (0.1 mg/ml, $P<0.01$), α_1M (0.25 mg/ml, $P<0.03$), α_1M (0.75 mg/ml, $P<0.01$), rat normal α_1M (1.5 mg/ml, $P<0.001$), and rat 5HT-α_1M (0.75 mg/ml, $P<0.001$).

1996a). In this study, we report that 5HT-α_1M, a monoamine-activated form of a rodent plasma protein homologue to human α_2M, has a similar long-term effect on the changes of CS DA concentrations. That is, rat 5HT-α_1M induces an overall statistically significant reduction in DA concentrations from the CS of male adult rats following infusion (Fig. 1A). The mean value of DA concentrations of the 5HT-α_1M-treated sides decreased to as low as 57% compared with the contralateral PBS control sides of the same animals. This inhibitory effect of rat 5HT-α_1M was unlikely due to a) covalent reaction between DA and 5HT-α_1M, since 5HT-α_1M does not have active thioester bonds, and b) the nonspecific absorption of DA by 5HT-α_1M via electrostatic interactions, since normal α_2M (which is very similar to α_1M in structural and charge properties) and BSA (which is more anionic than either α_1M or α_2M) were not inhibitory (Hu et al., 1994).

The experiment was also designed to test the time course of this inhibitory effect of 5HT-α_1M on the changes of DA concentrations in CS and OT. The data show a reduction of CS DA concentrations by 5HT-α_1M. This reduction appeared to have started from post-infusion day 2 and became more evident between day 4 and day 7 post-infusion, since there is no significant difference between individual readings of the CS samples of 5HT-α_1M-treated sides (Fig. 1A). The data failed to show any significant difference between the control and 5HT-α_1M-treated sides during post-infusion days 1 and 3. This absence of a statistically significant difference during this period is due to the reduced DA concentrations of the control sides, which may likely reflect a generalized recovery from the surgical lesions during this acute post-operative period. Variations in OT DA concentrations as a function of day of sacrifice may similarly be attributable to a generalized recovery from surgery. No significant differences between the DA concentrations between two sides of OT tissue samples taken from the same group of rats were observed on any of the post-infusion days tested (Fig. 1B). These OT data serve as an important control with regard to two aspects of our findings. First, they indicate that differences between the sampling of the two sides of the CS cannot be attributed to methodological factors involved in tissue dissection and assay. Second, they demonstrate the localized effect and the absence of diffusion of this protein with our infusions.

Our earlier preliminary results showed that rat 5HT-α_2M not only inhibited neurite outgrowth but also ChAT activity in embryonic rat basal forebrain cells in culture (Koo et al., 1994). In the present report we further show that rat MN-α_2M, like human MN-α_2M (Liebl and Koo, 1994), dose-dependently inhibited ChAT activity in these cells, whereas N-α_2M is inactive (Fig. 2). This inhibition is not due to loss of cell survival in culture, since the inhibition results were compared to the N-α_2M and BSA controls (Fig. 2) and these forebrain cells did not show significant reductions in number after 3 days of culture (Liebl and Koo, 1994). In addition, activated α_2M has been shown not directly toxic to other neurons, pheochromocytoma PC12 cells, and human neuroblastoma SY5Y cells (Koo and Liebl, 1992; Koo and Qiu, 1994; Hu, 1997). It is not surprising that both human and rat MN-α_2M should have identical suppressive effects on ChAT (Fig. 2) and neurite outgrowth (Koo and Liebl, 1992; Liebl and Koo, 1993a; Koo et al., 1994), since these macroglobulins are very similar antigenically, structurally as well as functionally. If α_2M is an important physiological and neurological regulator, it makes sense that it should be represented either at a high constitutive level in higher mammals such as humans (i.e., about 2 mg/ml in human serum) or as an acute-phase protein in some animals in response to stimuli. Apparently α_1M replaces α_2M in rats as a potential physiological and

neurological regulator under normal unstimulated conditions. If α_1M is to replace α_2M as a neuro-regulator, one would expect MN-α_1M should also be inhibitory to neurons; this is in fact what was found with regard to their effects on DA and ChAT (Figs. 1, 3). But the surprising finding is that N-α_1M is also dose-dependently inhibitory to ChAT (Fig. 3), whereas both rat and human N-α_2Ms are not (Fig. 2). Although α_1M is less inhibitory than MN-α_2M, it nevertheless could be sufficiently inhibitory to serve as a negative regulator for the ChAT activity in CNS neurons.

Rat α_1M and α_2M may potentially be involved in neuroregulation and neurodegeneration. Rat α_2M and its receptor (α_2MR/LRP) have been shown to occur in various tissues including the brain (Kodelja et al., 1986; Bu et al., 1994; Higuchi et al., 1994), and rat α-macroglobulins, like human α_2M, can noncovalently bind to, and potentially stabilize, a variety of neurotrophins like nerve growth factor (NGF) and brain-derived growth factor (BDNF) (Koo and Stach, 1989; Liebl, 1994). Not only these properties of rat α_2M are shared by the human, but human MN-α_2M are known also to exert significant effects on both PNS and CNS neurons (Koo and Liebl, 1992; Liebl and Koo, 1993a; Liebl and Koo, 1994; Koo and Qiu, 1994; Hu et al., 1996b; Çavuş et al., 1996). Since rat α-macroglobulins are structural and functional homologues of human α_2M, it is reasonable to assume that both rat α_1M and α_2M can also exert substantial influences upon the nervous system. Our recent studies with rat α-macroglobulin isoforms have indeed uncovered some fascinating effects of these proteins (Liebl, 1994; Koo et al., 1996). In the peripheral nervous system (PNS), rat α_1M and rat α_2M have been demonstrated to have opposing effects on sciatic nerve axonal regeneration, with 5HT-α_1M stimulating axonal regeneration while 5HT-α_2M inhibiting nerve regeneration. Hence rat α-macroglobulins, as a yin-yang pair, can potentially regulate the growth and regeneration of peripheral nerves. In the central nervous system (CNS), the effect of these two rat alpha-macroglobulins seems more complicated. The same opposing effects have been observed on neurite outgrowth in the embryonic rat basal forebrain cell cultures; for example, rat 5HT-α_2M, like human monoamine-activated α_2M, inhibited neurite extension, while 5HT-α_1M stimulated this process (Liebl and Koo, 1993a, Liebl, 1994). But both rat 5HT-α_1M and 5HT-α_2M produce a similar inhibitory effect on the neurotransmitter systems studied here.

The data presented here clearly show that both 5HT-α_1M and 5HT-α_2M can significantly inhibit choline acetyltransferase (CHAT) activities in embryonic basal forebrain cholinergic neurons in vitro. In addition, 5HT-α_1M can dramatically affect the DA levels in CS in a long time-course manner similar to that observed with human MA-α_2M (Hu et al., 1994). From this and our previous studies, one general principle appears to emerge. The activated form of α_2M, regardless the species of origin, is consistently inhibitory to various neuronal functions in both CNS and PNS, whereas rat α_1M is able to elicit different effects in neurons. Specifically, activated rat α_1M stimulated neurite outgrowth in vitro and sciatic nerve regeneration in vivo but inhibited neurotransmitter synthesis in the CNS. In this respect, α_1M behaves like growth factors or cytokines such as fibroblast growth factor-II (FGF-II) and interleukin 6 (IL-6). FGF-II supports neuronal survival and the growth of neurites in culture (Morrison et al., 1986; Walicke et al., 1986) but suppresses NGF-induced ChAT activity in embryonic septal cholinergic neurons (Matsuda et al., 1990). IL-6 is believed to accelerate the regeneration process of axotomized hypoglossal nerve in mice (Hirota et al., 1996) but also promotes cerebral degeneration in transgenic mice expressing higher levels of IL-6 (Campbell et al., 1993). Hence α-macroglobulins, like numerous cytokines capable of eliciting divergent effects from different tissues, may also play a regulatory role in nerve growth, regeneration, and neurotransmitter metabolism.

Basal forebrain cholinergic neurons and striatal dopamine neurons studied here represent major sites of degeneration in Alzheimer's and Parkinson's diseases, respectively, and an acute-phase state or the inflammatory process has been implicated as a major cause of such neurodegenerative diseases (Giometto et al., 1988; Abraham, 1992; Bauer et al., 1992; Stewart et al., 1997). Increased level of α_2M also appears to be associated with a) advancing age (Garton et al., 1991), b) stimulation of inflammatory cytokines (Ganter et al., 1991; Fabrizi et al., 1994; Hocke et al., 1992), c) Alzheimer's senile plaques (Van Gool et al., 1993), and d) an acute-phase state (Bauer et al., 1991, 1992). Here we demonstrate the effect of α-macroglobulins on the striatal and cholinergic neurons, and suggest a potentially important role for the α-macroglobulin family proteins in CNS neurodegenerative disorders associated with these systems.

Activated or ''fast'' α_2M has been detected to occur normally in various biologic fluids and its concentration is greatly increased in several human inflammatory conditions investigated (Jespersen et al., 1993; Bank et al., 1990, 1991). In addition, accumulation of amines has been detected in cerebral infarction, Parkinson's disease, Alzheimer's disease, and Huntington Chorea, as well as in the axons of degenerating neuros (Willis et al., 1987). The conditions under which activated α_2M can accumulate in the brain have been discussed (Hu et al., 1994). The locally accumulated α_2M, as detected in the senile plaques and Alzheimer's temporal cortex (Van Gool et al., 1993; Wood et al., 1993), can potentially react with and be activated by inflammatory amines and amino-

neurotransmitters generated in the CNS. According to our previous findings, the activated or ''fast'' α_2M is expected to more efficiently bind or ''entrap'' neurotrophins like NGF, BDNF, neurotrophin-3 (NT-3), and NT4/5 (Liebl and Koo, 1993b; Hu et al., 1994; Koo and Qiu, 1994; Wolf and Gonias, 1994), and activated α_2M can also selectively bind to *trk* receptors and inhibit neuronal functions (Koo and Qiu, 1994; Hu et al., 1996b; Koo et al., 1996). Via such interactions, activated α_2M may additionally decrease the concentrations of neurotransmitters and neurotrophins/cytokines in the vicinity, and may contribute to neuronal dysfunction.

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REFERENCES

Abraham CR (1992): The role of acute-phase protein α_1-antichymotrypsin in brain dysfunction and injury. Res Immunol 143:631–636.

Bank RE, Evans SW, Alexander D, Van Leuven F, Whicher JT, McMahon MJ (1991): Alpha-2-macroglobulin state in acute pancreatitis. Raised values of alpha-2-macroglobulin-protease complexes in severe and mild attacks. Gut 32:430–434.

Bank RE, Evans SW, Van Leuven F, Alexander D, McMahon MJ, Whicher JT (1990): Measurement of the ''fast'' or complexed form of alpha-2-macroglobulin in biological fluids using a sandwich enzyme immunoassay. J Immunol Methods 126:13–20.

Barrett AJ, Brown MA, Sayers CA (1979): The electrophoretically ''slow'' and ''fast'' forms of the alpha-2-macroglobulin molecule. Biochem J 181:401–418.

Bauer J, Ganter U, Strauss S, Stadtmuller G, Frommberger U, Bauer H, Volk B, Berger M (1992): The participation of IL-6 in the pathogenesis of Alzheimer's disease. Res Immunol 143:650–657.

Bauer J, Strauss S, Schreiter-Gasser U, Ganter U, Schlegel P, Witt I, Volk B, Berger M (1991): Interleukin-6 and α-2-macroglobulin indicate an acute-phase state in Alzheimer's disease cortices. FEBS Lett 285:111–114.

Bu G, Masksymovitch EA, Nerbonne JM, Schwartz AL (1994): Expression and function of the low density lipoprotein receptor-related protein (LRP) in mammalian central neurons. J Biol Chem 269:18521–18528.

Campell IL, Abraham CR, Masliah E, Kemper P, Inglis JD, Oldstone MB, Mucke L (1993): Neurologic disease induced in transgenic mice by cerebral overexpression of interleukin 6. Proc Natl Acad Sci USA 90:10061–5.

Çavuş I, Koo PH, Teyler TJ (1996): Inhibition of long-term potentiation development in rat hippocampal slice by α_2-macroglobulin, an acute phase protein in the brain. J Neurosci Res 43:282–288.

Eggertsen G, Hudson G, Shiels B, Reed D, Fey GH (1991): Sequence of rat α_1-macroglobulin, a broad-range proteinase inhibitor from the α-macroglobulin-complement family. Mol Biol Med 8:287–302.

Fabrizi C, Colasanti M, Persichini T, Businaro R, Starace G, Lauro GM (1994): Interferon gamma up-regulates α_2-macroglobulin expression in human astrocytoma cells. J Neuroimmunol 53:31–37.

Fonnum F (1975): A rapid radiochemical method for the determination of choline acetyltransferase. J Neurochem 24:407–409.

Ganrot K (1973): Rat α_2-acute phase globulin, a human α_2-macroglobulin homologue: Interaction with plasmin and trypsin. Biochim Biophys Acta 322:62–67.

Garton MJ, Keir G, Lakshmi MV, Thompson EJ (1991): Age-related changes in cerebrospinal fluid protein concentrations. J Neurol Science 104:74–80.

Ganter U, Strauss S, Jonas U, Weidemann A, Beyreuther K, Volk B, Berger M, Bauer J (1991): Alpha-2-macroglobulin synthesis in interleukin-6-stimulated human neuronal(SH-SY5Y neuroblastoma) cells. FEBS Lett 282:127–131.

Giometto B, Argentiero V, Sanson F, Ongaro G, Tavolato B (1988): Acute phase proteins in Alzheimer's disease. Eur Neurol 28:30–33.

Gordon AH (1976): The α_2-macroglobulins of rat serum. Biochem J 159:643–650.

Higuchi M, Ito T, Imai Y, Iwaki T, Hattori M, Kohsaka S, Niho Y, Sakaki Y (1994): Expression of the α_2-macroglobulin-encoding gene in rat brain and cultured astrocytes. Gene 141:155–162.

Hirota H, Kiyama H, Kishimoto T, Taga T (1996): Accelerated nerve regeneration in mice by upregulated expression of interleukin (IL)-6 and IL-6 receptor after trauma. J Exp Med 183:2627–34.

Hocke GM, Cui MZ, Ripperger JA, Fey GH (1992): Regulation of the rat α_2-macroglobulin gene by interleukin-6 and leukemia inhibitory factor. Folia Histochemi Cytobiol 30:137–139.

Hu YQ (1997): The effect of alpha-2-macroglobulin on dopamine concentration in corpus striatum and *trk*B-mediated signal transduction and gene expression: A study on its potential role in neurodegeneration in the central nervous system. Ph.D. dissertation, The Kent State University/Northeastern Ohio Universities College of Medicine.

Hu YQ, Dluzen DE, Koo PH (1994): Intracranial infusion of monoamine-activated α_2-macroglobulin decreases dopamine concentrations within the rat caudate putamen. J Neurosci Res 38:531–537.

Hu YQ, Liu BJ, Dluzen DE, Koo PH (1996a): Alterations of dopamine release by rat caudate putamen tissues superfused with α_2-macroglobulin. J Neurosci Res 43:71–77.

Hu YQ, Zhang J, Koo PH (1996b): Activated α_2-macroglobulin blocks signal transduction mediated by neurotrophic receptors, *trk* B and *trk*C. FASEB J 10:A1407.

Hudson NW, Koo PH (1982): Subunit and primary structure of a mouse alpha-macroglobulin, a human α_2-macroglobulin homologue. Biochim Biophys Acta 704:290–303.

Jespersen MH, Jensen J, Rasmussen LH, Ejlersen E, Moller-Petersen J, Sperling-Petersen HU (1993): The reference range for complexed α_2-macroglobulin in human plasma: Development of a new enzyme linked in immunosorbent assay (ELISA) for quantitation of complexed α_2-macroglobulin. Scand J Clin Lab Invest 53:639–648.

Kodelja V, Heisig M, Northemann W, Heinrich PC, Zimmermann W (1986): Alpha-2-macroglobulin gene expression during rat development studied by in situ hybridization. EMBO J 5:3151–3156.

Koo PH, Lee PG, Liebl DJ, Witt WC (1996): Rat α_1- and α_2-macroglobulin respectively enhance, and inhibits trkA activation and sciatic nerve regeneration. Neuroimmunomodulation 3:186.

Koo PH, Liebl DJ (1992): Inhibition of nerve growth factor-stimulated neurite outgrowth by methylamine-modified α_2-macroglobulin. J Neurosci Res 31:678–692.

Koo PH, Liebl DJ, Qiu WS, Hu YQ, Dluzen DE (1994): Monoamine-activated α_2-macroglobulin inhibits neurite outgrowth, survival, choline acetyltransferase, and dopamine concentration of neurons by blocking neurotrophin-receptor (*trk*) phosphorylation and signal transduction. Ann N Y Acad Sci 737:460–464.

Koo PH, Qiu WS (1994): Monoamine-activated α_2-macroglobulin binds *trk* receptor and inhibits nerve growth factor-stimulated *trk* phosphorylation and signal transduction. J Biol Chem 269:5369–5376.

Koo PH, Stach RW (1989): Interaction of nerve growth factor with murine alpha-macroglobulin. J Neurosci Res 22:247–261.

Liebl DJ (1994): The regulation of neurite outgrowth and neuronal survival by α_2-macroglobulin homologues. Ph.D. dissertation, Kent State University/Northeastern Ohio Universities College of Medicine.

Liebl DJ, Koo PH (1993a): Serotonin-activated α_2-macroglobulin inhibits neurite outgrowth and survival of embryonic sensory and cerebral cortical neurons. J Neurosci Res 35:170–182.

Liebl DJ, Koo PH (1993b): Comparative binding of neurotrophins (NT-3, CNTF and NGF) and various cytokines to alpha-2-macroglobulin. Biochem Biophys Res Commun 193:1255–1261.

Liebl DJ, Koo PH (1994): Monoamine-activated α_2-macroglobulin inhibits choline acetyltransferase of embryonic basal forebrain neurons and reversal of the inhibition by NGF and BDNF but not NT-3. J Neurosci Res 38:407–414.

Lonberg-Holm K, Reed DL, Roberts RC, Damato-McCabe D (1987a): Three high-molecular-weight protease inhibitors of rat plasma. Reactions with trypsin. J Biol Chem 262:4844–4853.

Lonberg-Holm K, Reed DL, Roberts RC, Hebert RR, Hillman MC, Kutney RM (1987b): Three high-molecular-weight protease inhibitors of rat plasma: Isolation, characterization, and acute phase changes. J Biol Chem 262:438–445.

Lowery OH, Rosebrough NJ, Farr AL, Randall RJ (1951): Protein measurement with folin phenol reagent. J Biol Chem 193:265–275.

Matsuda S, Saito H, Nishiyama N (1990): Basic fibroblast growth factor suppressed the enhancement of choline acetyltransferase activity induced by nerve growth factor. Neurosci Lett 114:69–74.

Moestrup SK, Gliemann J, Pallesen G (1992): Distribution of the α_2-macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues. Cell Tissue Res 269:375–82.

Morrison RS, Sharma A, De Vellis J, Bradshaw RA (1986): Basic fibroblast growth factor supports the survival of cerebral cortical neurons in primary culture. Proc Natl Acad Sci USA 83:7537–7541.

Sand O, Folkersen J, Westergaard JG, Sottrup-Jensen L (1985): Characterization of human pregnancy zone protein: Comparison with human α_2-macroglobulin. J Biol Chem 260:15723–15735.

Schaeufele JT, Koo PH (1982): Structural comparison of rat α_1- and α_2-macroglobulins. Biochem Biophys Res Commun 108:1–7.

Sottrup-Jensen L, Petersen TE, Magnusson S (1981): Trypsin-induced activation of the thiolesters in alpha-2-macroglobulin generates a short-lived intermediate (''Nascent'' alpha-2-M) that can react rapidly to incorporate not only methylamine or putrescine but also proteins lacking proteinase activity. FEBS Lett 128:123–126.

Stewart WF, Kawas C, Corrada M, Metter EJ (1997): Risk of Alzheimer's disease and duration of NSAID use. Neurology 48:626–632.

Strauss S, Bauer J, Ganter U, Jonas U, Berger M, Volk B (1992): Detection of interleukin-6 and α_2-macroglobulin immunoreactivity in cortex and hippocampus of Alzheimer's disease patients. Lab Invest 66:223–230.

Swenson R, Howard JB (1979): Structural characterization of human α_2-macroglobulin. J Biol Chem 254:4452–4456.

Van Gool D, DE Strooper B, Van Leuven F, Triau E, Dom R (1993): Alpha-2-macroglobulin expression in neuritic-type plaques in patients with Alzheimer's disease. Neurobiol Aging 14:233–237.

Walicke PA, Cowan WM, Ueno N, Baird A, Guillemin R (1986): Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. Proc Natl Acad Sci USA 83:3012–3016.

Willis GL (1987): Amine accumulation in Parkinson's disease and other disorders. Neurosci Biobehav Rev 11:97–105.

Wolf BB, Gonias SL (1994): Neurotrophin binding to human α_2-macroglobulin under apparent equilibrium conditions. Biochemistry 33:11270–11277.

Wood JA, Wood PL, Ryan R, Graff-Radford NR, Pilapil C, Robitaille Y, Quirion R (1993): Cytokine indices in Alzheimer's temporal cortex: no changes in mature IL-1-beta or IL-1RA but increases in the associated acute phase proteins IL-6, alpha-2-macroglobulin and C-reactive protein. Brain Res 629:245–252.